substitutions based on the scoring matrix were chosen at each of these sites. This library was termed the 'profile library' or IRL1 library.

To create the IRL1 DNA library, 90 mutagenic forward primers containing the different substitutions were designed and used in a PCR reaction containing a single wild type reverse primer and the *E. cloacae ampC*-containing plasmid pAL20 as template. After digestion of the methylated template DNA using the *DpnI* enzyme, the PCR product was used to transform *E.coli*. The transformants were plated on kanamycin plates to determine the number of transformants obtained or kanamycin plates containing different concentrations of moxalactam (mox) to obtain moxalactam resistant clones. The mox-resistant clones were further characterized to determine the fold increase in resistance compared to cells containing the wild type ampC gene. Ten mox-resistant clones were obtained, which had a fold increase in mox-resistance ranging from around 3-fold to 20-fold (0.8-6 μg/mL) above wild type (0.3 μg/mL).

Sequencing of the *ampC* gene in the plasmids from these variants revealed that each of them contained one to three of the selected library amino acid changes in ampC (Table 4). Two of the variants, IRL1.8.4 and IRL1.8.5 also contained additional mutations introduced during the PCR process (Table 4). The IRL1.6.1 variant, which has a 20-fold increase in mox-resistance was the best variant in this library and had two changes at positions S288 and R348. The substitutions Y220N, A219P and L61M appeared in more than one clone suggesting that they may be important for conferring resistance. Thus, this example shows that the application of a distance-based constraint onto a scoring matrix was successful in producing ampC variants that had a significantly higher resistance to the antibiotic moxalactam.

<u>Example 3. Alteration of β-lactamase Specificity Using a Recruitment Matrix</u>

This Example demonstrates the application of a distance-based constraint vector to the *E. cloacae* ampC molecule and recruitment of amino acids observed in other ampC proteins.

To create the IRL library in this example, first, the sequence of the ampC protein from *E. cloacae* (reference sequence) was aligned with ampC protein sequences from *A. sobria*, *E. coli*, *O. anthropi*, *P. aeroginosa*, *S. enteriditis* and *Y. enterolitica* using the AlignX program from Vector NTI Suite (Informax Inc. Bethesda, MD). Those positions in the alignment where amino acids other than those found in the reference sequence were observed were recruited, and a distance-based constraint vector was applied to these positions to limit mutations to residues that

were surface exposed and 6 angstroms from the binding site of ligands to the *E. cloacae* ampC 3-D structure. Specifically, the *E. cloacae* ampC crystal structure (Protein Database Base ID# 1BLS) and 6 *E. coli* ampC structures containing bound inhibitors or substrates (Protein Database Base structures 1C3B, 1FCM, 1FCN, 1FCO, 1FSW, 1FSY) were first loaded into the program MOE 2000.01 (Chemical Computing Group, Inc., Montreal Canada). Because each structure consists of a homodimer, one of the monomers and its associated ligand was deleted. Next, the main chains of all the structures containing bound ligands were aligned (0.4 angstroms RMS deviation) and all the water molecules were manually deleted. The main chains of all structures except the *E. cloacae* structure (1BLS) were then removed. The resulting structure consisted of the *E. cloacae* ampC molecule with all of the superimposed ligands from the other 6 ampC structures. All surface-exposed side chains (i.e, did not count the backbone, just the beta carbon, and outward atoms) in ampC with atoms within 6 angstroms of the ligand atoms were then selected for the IRL library. Eight positions were selected and substitutions were chosen based on the amino acids observed at those positions in other members of the ampC protein family used in the alignment. This library was termed the 'recruitment library' or IRL2 library.

To create the IRL2 DNA library, 15 mutagenic forward primers containing the different substitutions were designed and used in a PCR reaction containing a single wild type reverse primer and the *E. cloacae ampC*-containing plasmid pAL20 as template. After digestion of the methylated template DNA using the *DpnI* enzyme, the unmethylated PCR product was used to transform *E.coli*. The transformants were plated on kanamycin plates to determine the number of transfomants obtained or kanamycin plates containing different concentrations of moxalactam (mox) to obtain moxalactam resistant clones. The mox-resistant clones were further characterized to determine the fold increase in resistance compared to cells containing the wild type ampC gene. Fifteen mox-resistant clones were obtained, which had a fold increase in mox-resistance ranging from around 3 fold to 83 fold (0.8-25 μg/mL) above wild type (0.3 μg/mL) in a single round.

Sequencing of the ampC gene in the plasmids from these variants revealed that 12 variants contained one to three of the desired library amino acid changes in ampC (Table 4). In addition to the desired mutations observed in the winners, some of the winners had additional unexpected mutations which may have contributed to the phenotype in some cases. Four of the variants contained additional unexpected mutations either in the promoter or within the ampC

gene due to errors in the PCR process. These included S263P in IRL1.8.4, S17T in the signal sequence in IRL1.8.5, A217V in IRL2.8.4, and T125M in IRL2.3.6. The observation that 3 of the 15 variants contained wild type ampC sequence suggests that mutations elsewhere in the plasmid vector or in the E. coli genome can contribute to the phenotype, which is not unexpected. Silent muations were also seen at position A351 in IRL1.8.10, S286 in IRL2.8.3, and at A152 in IRL2.8.14. Promoter region mutations were seen in IRL2.8.7 (a to g at +168), IRL2.8.12 (c to t at +136), and IRL2.8.13 (c to t at +237 and t to c at +205).

The substitutions V120F and N345I appeared in several clones suggesting their importance for increasing mox resistance. Although it can be argued that these mutations came up several times due to PCR primer bias, the sequencing of random library clones not selected for mox resistance did reveal other positions where a large number of substitutions were seen, but which did not show up in the variants. It is interesting that compared to the IRL1 library, the IRL2 library shows a different profile of substitutions in the variants. Again, this example shows that the use of a distance-based constraint and recruited residues from multiple sequence alignment were successful in producing ampC variants that had a significantly higher resistance to the antibiotic moxalactam.

Molecular Biological Methods

The mutagenic primers used for creating the PCR-based DNA libraries each contained 37 bases with 17 bases flanking the mutant codon on both sides. All mutagenic and wt primers used for creating the DNA libraries or for sequencing were obtained from Operon Technologies (Alameda, CA).

A single reverse primer and 90 IRL1 or 15 IRL2 mutagenic forward primers were used in a PCR reaction with a template, plasmid pAL20 containing the *E. cloacae ampC* gene. Plasmid pAL20 was created by sub-cloning the *ampC* gene into the TOPOBLUNT vector (*kan*^{r}) obtained from Invitrogen (Carlsbad, CA). The final reaction contained 0.5μM of the reverse primer and 0.5μM of all IRL forward primers combined (all primers together were 25 pmols), 16 fmol of pAL20, 15 nmol of each dNTPs, 5 units of the Herculase polymerase (Stratagene, La Jolla, CA) and a Herculase-specific buffer also from Stratagene. The total reaction volume was 100μL. The cycling conditions included an initial cycle at 94°C for 3 minutes followed by 30 cycles each containing a step at 94°C for 30 seconds, a 55°C step for 30s and a 68°C step for 5